Screening for novel nucleic acid and peptide medicaments by selection method

Rika Yamamoto, Satoshi Fujita, P.K.R. Kumar, Kazunari Taira

Methods of screening for functional nucleic acids and functional peptides *in vitro* by molecular selection methods are being developed. A screening method for nucleic acids has already been established, and a novel functional RNA that inhibits transcription of HIV has been created by this method. This novel nucleic acid, binds with the Tat protein (transcription activating factor) of HIV with high affinity, and it is expected to be used as an anti-HIV agent having few side effects. With regard to peptides, as yet no outstanding screening method has been established, and at present various methods of screening for functional peptides which could contribute to development of peptide medicaments are being suggested.

Key Words: in vitro selection method, HIV, aptamer

Introduction

Nucleic acids were long believed to be no more than templates for proteins, however, in recent years, attention has been drawn to discoveries of molecules having functions such as nucleic acids having enzyme activity (ribozymes, DNA-zymes) and nucleic acids which bind to specific molecules (aptamers). Further, the existence of functional molecules among peptides, such as pepzymes has been confirmed. Further, a method of introducing genes to a human body using adenovirus vector has been developed, so the value of using these molecules as medicaments has dramatically increased. That is, it has become possible to express nucleic acids and peptides which function as medicaments from vectors introduced into the human body. Thus, the development of novel functional nucleic acids and peptides is very significant for the development of new medicaments. However, it is extremely difficult to search for compounds that can form the basis of new medicaments from nature. Thus an *in vitro* method for screening functional nucleic acids and functional peptides was developed. A novel functional RNA which inhibits HIV transcription that was obtained by this method binds to the HIV Tat protein with high affinity and thus is expected to be useful as an anti-HIV agent with few side-effects.

In this paper, we introduce a method for screening functional nucleic acids and functional peptides using a molecular selection method, as well as a novel functional RNA that inhibits transcription of HIV that was created by this method.

I. Nucleic Acid Medicament Development

1. Suitability of nucleic acid as a medicament

2. *In vitro* Selection Method

3. HIV Tat protein (p.(377)33, left column)

When human immunodeficiency virus (HIV) is transcribed from its provirus (where the virus gene is present on the chromosome of the host cell in the form of DNA), it exhibits explosive growth in the presence of a particular protein. The protein which plays this important role is called *trans*-activating protein (Tat). It binds specifically to a region which adopts a stem-loop structure called the TAR (*trans*-activating response region) which is present on the initial transcription product from LTR of HIV, and it activates transcription which has halted at the TAR region and assist further transcription. The difference in expression level as between when Tat protein is present and when Tat protein is absent is said to be by a factor of 100 to 1000. Further, apart from transcription, the Tat protein is reported to play an important role in reverse transcription. Therefore, if the operation of this protein can be inhibited, there is a possibility that this will more efficiently suppress the proliferation of the virus than would inhibition of reverse transcriptases or proteases. Further, a deficiency of presently known anti-viral agents is that a virus having resistance to the agent soon appears. However, the conserved region of the Tat protein is essential to the virus, and is a site in which mutation rarely occurs. Thus it could be unlikely that a resistant virus would appear.

4. Comparison of affinity for Tat protein between Tat aptamer and TAR RNA.

From among a group of RNA molecules obtained by an *in vitro* selection method, several sequences were examined at random. Approximately 40% of the RNA molecules comprised the conserved sequence (TAR core sequence, Figure 2-a) which is essential for TAR RNA binding to Tat protein. Further, interestingly, among these RNA molecules, molecules having two neighboring TAR core sequences were found (Fig 2-b). When the binding abilities of these respective RNA molecules were examined, because the binding ability of RNA molecules having a stem-loop structure comprising two TAR core sequences was greater, we chemically synthesized only the stem-loop structure portion of these RNA molecules, and designated it a Tat aptamer (Fig. 2-c). This Tat aptamer in comparison with TAR RNA, exhibits high binding ability at

extremely low concentrations. Further, using gel shift method in a similar manner, we directly compared the affinity to Tat protein of TAR RNA and Tat aptamer, and found that our aptamer exhibited affinity approximately 100 times greater than TAR RNA.

5. Application of Tat aptamer to HIV-2 (Human Immunodeficiency Virus Type 2)

No method of treatment has been established for HIV-2, which, while there are few cases in developed countries, is prevalent mainly in West Africa. The Tat protein and TAR region of HIV-2 bear little resemblance to those of HIV-1, however, an arginine-rich site which is necessary for interaction with TAR in the Tat protein is conserved to some degree, and the TAR core sequence present in the TAR region is common between the two. Thus, we examined what kind of properties our Tat aptamer had in respect of the Tat protein of HIV-2. As a result, we found that Tat aptamer, had affinity approximately 50 times greater than HIV-2 TAR in respect of HIV-2 Tat derived peptide also. Therefore, it is indicated that our Tat aptamer may also be applied to HIV-2.

6. Presence or absence of side effects of Tat aptamer

It is clear that when transcription is promoted by Tat protein, the transcription factor of the host cell (polymerase II, TRP-185, etc.) binds to the TAR region. It has been shown that TAR RNA inhibits transcription from promoters other than that of HIV unrelated to Tat/TAR interactions. This suggests that when TAR RNA is used as a Tat protein inhibitor, as a side effect, there is the possibility that TAR RNA non-selectively influences transcription other than that of the virus such as transcription of house-keeping genes. However, in an nuclear extract of HeLa cells, in the presence of the above described Tat aptamer, transcription from promoters other than that of HIV was not inhibited.

From the above it was clear that our aptamer was extremely promising as a medicament that selectively inhibits the action of Tat protein, and thereby suppresses proliferation of the virus. Further, the strength of the binding affinity of this aptamer for Tat protein was greater than any protein-nucleic acid interaction known to date, and so applications therefor are anticipated. In Section II of this paper, an example of using the aptamer as a tool for screening for functional peptides is presented.

II. Development of a peptide medicament

1. Suitability of peptides as medicaments

2. *In vitro* Selection Methods for peptides

3. Peptide *in vitro* selection method using interaction between Tat and Tat aptamer (p.(379)35, central column)

Presently, we are attempting to develop a novel peptide *in vitro* selection method. That we found a Tat aptamer which binds with Tat protein with high affinity is as described above. In our method, we use a peptide being the binding domain portion of Tat (Tat peptide) and Tat aptamer for a link between mRNA and a peptide. The major advantages of this method are that (1) since the tools used in linkage are all pure RNA or protein, there is no need for a synthesis step in the cycle, and (2) the mRNA and peptide are directly linked via Tat peptide and RNA (Tat) aptamer. However, since linking is not via covalent bonds as in the puromycin method, the key to success will be the raising of affinity to a level where disassociation does not occur. Thus, we attempted to strengthen binding between mRNA and the peptide by allowing multiple linkages in tandem between the Tat peptide and Tat aptamer. (Fig 3-3). The authors are aware of several reports of increasing affinity by linking a plurality of binding domains. Further, even in nature, there are not a few examples of functional molecules having a plurality of binding domains to increase certainty of binding. This new *in vitro* selection method, just as with the ribosome display method and puromycin method involves selection of functional molecules from various peptide molecules by repeating a cycle of transcription→translation→selection→reverse transcription→amplification. An mRNA encoding a peptide being a subject of selection and Tat peptides and Tat aptamers is transcribed *in vitro* with double-stranded DNA as a template, and translated. Whereupon, the mRNA comprising the Tat aptamer and the Tat peptide of the fusion peptide rapidly bind and a complex of a peptide and an mRNA encoding the peptide is formed. By performing selection in respect of this complex, a peptide having function of interest is selected, and this information is obtained by reverse transcription of mRNA and PCR amplification.

Conclusion

Various types of functional nucleic acids are being created by selection methods, but for these to be actually used as medicaments in subjects, there are many varied problems such as the establishment of stability, control of expression level, safety of vectors used in introduction. In this stage of development of means for creating function peptides, the development of a superior screening method is desired.

Figure 1: *In vitro* Selection Method

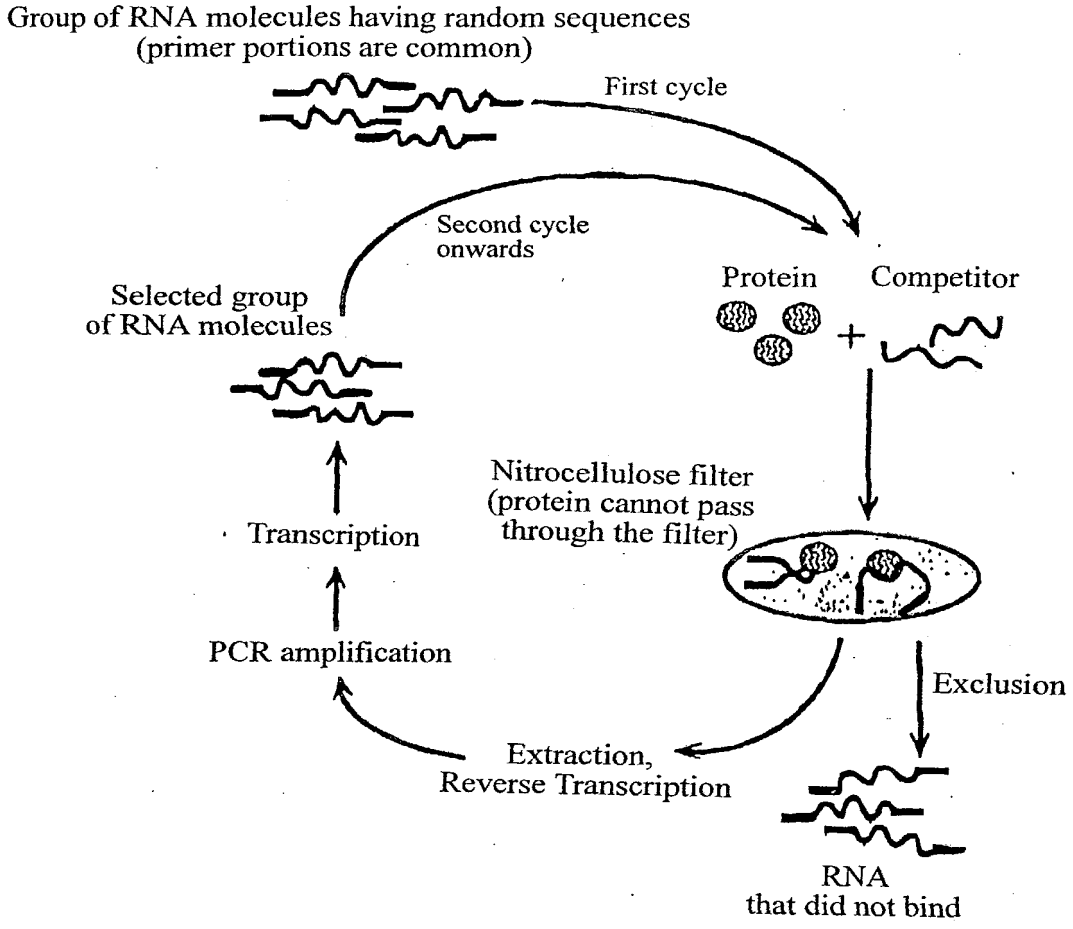
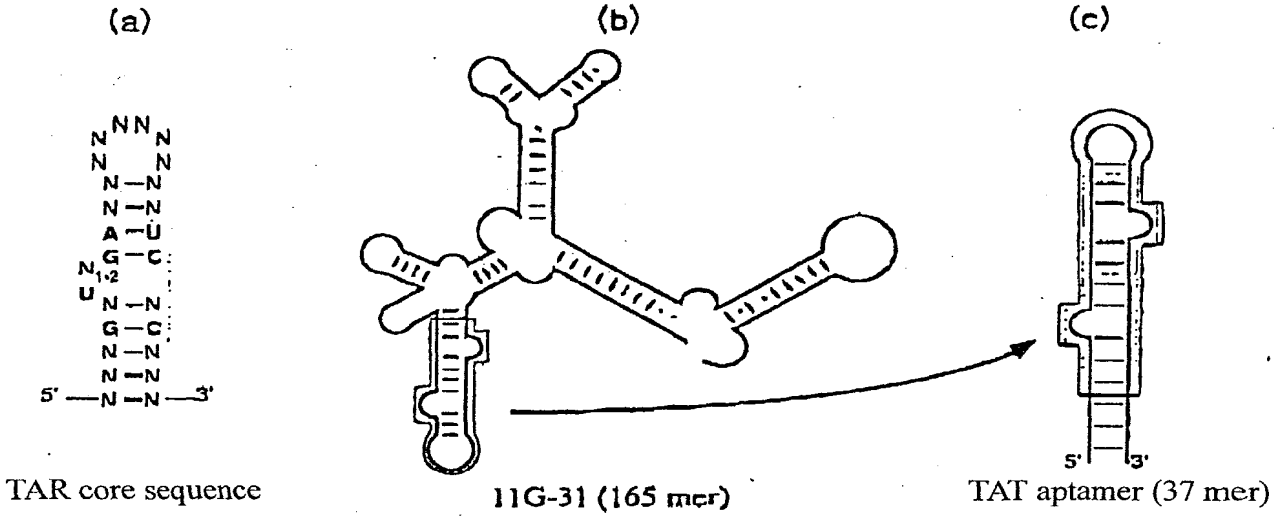


Figure 2: TAR core sequence and RNA obtained by *in vitro* selection



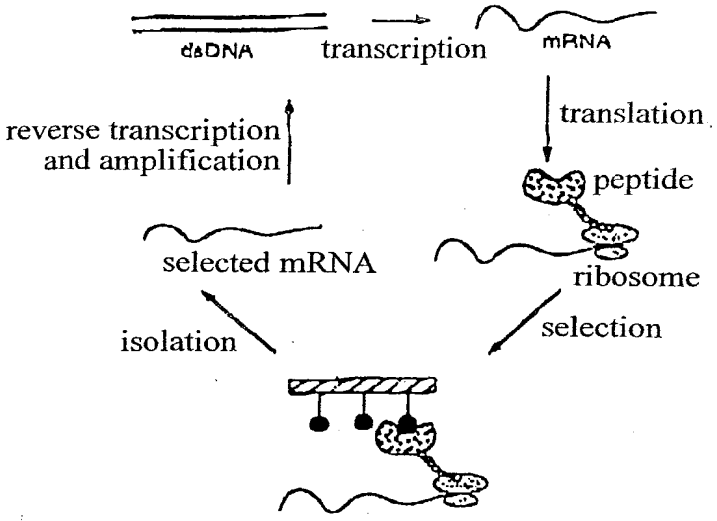
TAR core sequence

11G-31 (165 mer)

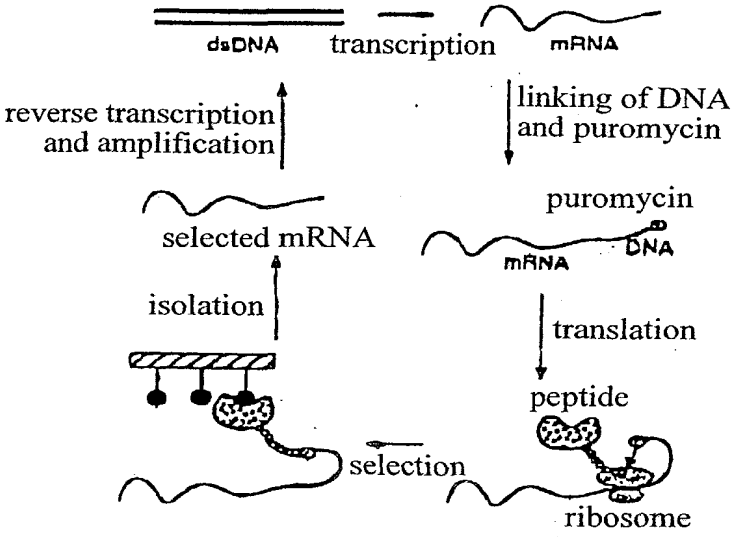
TAT aptamer (37 mer)

Figure 3: Peptide (Protein) *in vitro* selection methods

1. Ribosome display method



2. Puromycin linking method



3. Tat aptamer linking method